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STATE-OF-THE-ART REVIEW

The role of RNA-binding proteins in mediating adaptive responses in Gram-positive bacteria

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Keywords

adaptive responses; bacteria; gram-negative; gram-positive; protein–RNA interactions; RNA-binding proteins

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Bacteria are constantly subjected to stressful conditions, such as antibiotic exposure, nutrient limitation and oxidative stress. For pathogenic bacteria, adapting to the host environment, escaping defence mechanisms and coping with antibiotic stress are crucial for their survival and the establishment of a successful infection. Stress adaptation relies heavily on the rate at which the organism can remodel its gene expression programme to counteract the stress. RNA-binding proteins mediating co- and post-transcriptional regulation have recently emerged as important players in regulating gene expression during adaptive responses. Most of the research on these layers of gene expression regulation has been done in Gram-negative model organisms where, thanks to a wide variety of global studies, large post-transcriptional regulatory networks have been uncovered. Unfortunately, our understanding of post-transcriptional regulation in Gram-positive bacteria is lagging behind. One possible explanation for this is that many proteins employed by Gram-negative bacteria are not well conserved in Gram-positives. And even if they are conserved, they do not always play similar roles as in Gram-negative bacteria. This raises the important question whether Gram-positive bacteria regulate gene expression in a significantly different way. The goal of this review was to discuss this in more detail by reviewing the role of well-known RNA-binding proteins in Gram-positive bacteria and by highlighting their different behaviours with respect to some of their Gram-negative counterparts. Finally, the second part of this review introduces several unusual RNA-binding proteins of Gram-positive species that we believe could also play an important role in adaptive responses.

Introduction

Co- and post-transcriptional regulation involves control of transcription, translation efficiency and mRNA transcript stability. RNA-binding proteins (RBPs) are fundamental components of bacterial co- and post-transcriptional regulatory networks. They can influence transcription and translation in many ways. For example, they can block translation by binding ribosome-binding sites, and they can trigger transcription

termination or promote transcription elongation by altering the structure of the mRNA (attenuation and antitermination, respectively). Our understanding of the antitermination and attenuation mechanisms in bacteria has been facilitated by research on various RBPs, such as the Bgl-Sac protein family in *Escherichia coli* and *Bacillus subtilis* and the TRAP attenuation protein of *B. subtilis* as recently described in a very informative

Abbreviations

(p)ppGpp, guanosine pentaphosphate; CLASH, cross-linking, ligation and sequencing of hybrids; CRAC, cross-linking and analysis of cDNAs; CSD, cold-shock domain; CSP, cold-shock protein; DRBP, DNA- and RNA-binding protein; IFN, interferon; RBP, RNA-binding protein; SAS, small alarmone synthetase; SD, Shine–Dalgarno; sRNA, small RNA.

review [1]. In addition, RBPs often act in cooperation with small RNAs (sRNAs) to manipulate the function of their target molecules, by guiding sRNA–RNA interactions or recruiting RNA degradation factors to control RNA degradation rates [2,3].

Technological advances in studying protein–RNA and RNA–RNA interactions revealed that bacteria express many more RBPs than expected and unearthed huge networks of sRNA–RNA interactions that link diverse cellular pathways [4–16]. A surprising finding from these high-throughput proteomic studies was the sheer abundance of unconventional RBPs, including metabolic enzymes, such as aconitase (IRP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In many cases are these proteins and their RNA-binding activity conserved in bacteria [11,12,17–19]. Since a number of reviews have recently been published that discuss the findings from these high-throughput studies in detail [20–30], we will not further discuss this here. Additionally, the roles of many well-characterized bacterial RBPs, including RNA decay factors, have recently been thoroughly reviewed [3,9,31–36].

Here, we mainly focus on the role of RBPs in regulating gene expression in Gram-positive bacteria, as these are relatively understudied. In the first part of the review, we discuss a number of conserved RBPs and, where possible, compare their role in Gram-positives to their Gram-negative counterparts. It is becoming increasingly clear that Gram-positive bacteria may, in some ways, ‘do things differently’ when it comes to regulation of gene expression. What do we mean with ‘doing things differently’? For example, even though many well-studied RBPs of Gram-negative bacteria are conserved in Gram-positives, these proteins do not always seem to contribute to post-transcriptional regulation in the same way or to the same extent. One goal of this review was to shed light on some of these differences.

As stated above, many of the novel RBPs that were recently identified do not contain conventional RNA-binding domains and we would not be surprised if these types of RBPs will be much more in the limelight in the near future. Therefore, the second part of this review focusses on three atypical RBPs that we believe deserve more attention.

The role of well-known RBPs in regulating adaptive responses in Gram-positive bacteria

Hfq only plays important roles in some Gram-positive bacteria

Near-universal bacterial RBP Hfq and Gram-negative-specific ProQ have been shown to play a very

important role in co- and post-transcriptional gene regulation in Gram-negative bacteria by facilitating sRNA–mRNA interactions and controlling the stability and translation of transcripts [6,37–43]. Both proteins have been extensively studied in Gram-negative bacteria and were shown to regulate bacterial virulence and adaptation to stress (reviewed in detail in Ref. [37,38,44,45]). Hfq is an Sm-like RBP that plays an important role in mediating base-pairing interactions between many sRNAs and their RNA substrates. Hfq forms homohexameric rings (Fig. 1A) that have many surfaces for binding RNAs in a sequence-specific manner, including the lateral/rim, distal and proximal regions and the C-terminal tail. The distal face of Hfq binds mRNA A-rich sequences, while the proximal face binds A/U-rich sequences and sRNAs [46]. The efficiency of the Hfq chaperone activity was shown to depend on the number of arginine residues in the Hfq rim motif (RRER in *E. coli*; Fig. 1; [47]). ProQ is another Gram-negative RNA chaperone protein that, like Hfq, plays an important role in mediating RNA–RNA interactions. Unlike Hfq, ProQ is a monomeric protein that has preference for structured RNAs [6,41]. However, in Gram-positive bacteria, ProQ is not present, and the function of Hfq in Gram-positives appears to be somewhat controversial (discussed below). This is surprising, given that these two proteins are such big players in post-transcriptional regulation in Gram-negative bacteria.

What could be the explanation for this? Hfq is absent in several low GC Gram-positive bacteria, including *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Enterococcus faecium* [48] (see Table 1 for GC content). Interestingly, those proteobacteria where Hfq plays an important function, such as *E. coli* and *Salmonella enterica*, have a relatively high GC content ($\geq 50\%$), especially compared with Gram-positive bacteria ($\sim 32\%$ in *Staphylococcus aureus*; see Table 1) [49]. Thus, it was proposed that the involvement of Hfq in sRNA-mediated regulation is linked to the GC content of the genome and its function is most important for high GC content organisms. A model was proposed where strong sRNA–mRNA interactions that occur due to the high GC content need to be loosened by a protein chaperone, so that a functional regulatory interaction is produced [49]. High GC content can also lead to stable intramolecular sRNA or mRNA structures that may need to be relaxed to allow optimal regulation. For example, some mRNAs form inhibitory hairpins at their 5' end. Hfq has been shown to be necessary for relieving these structures and promoting the interactions with regulatory sRNAs [50]. Similarly, RNA interference in eukaryotes is more

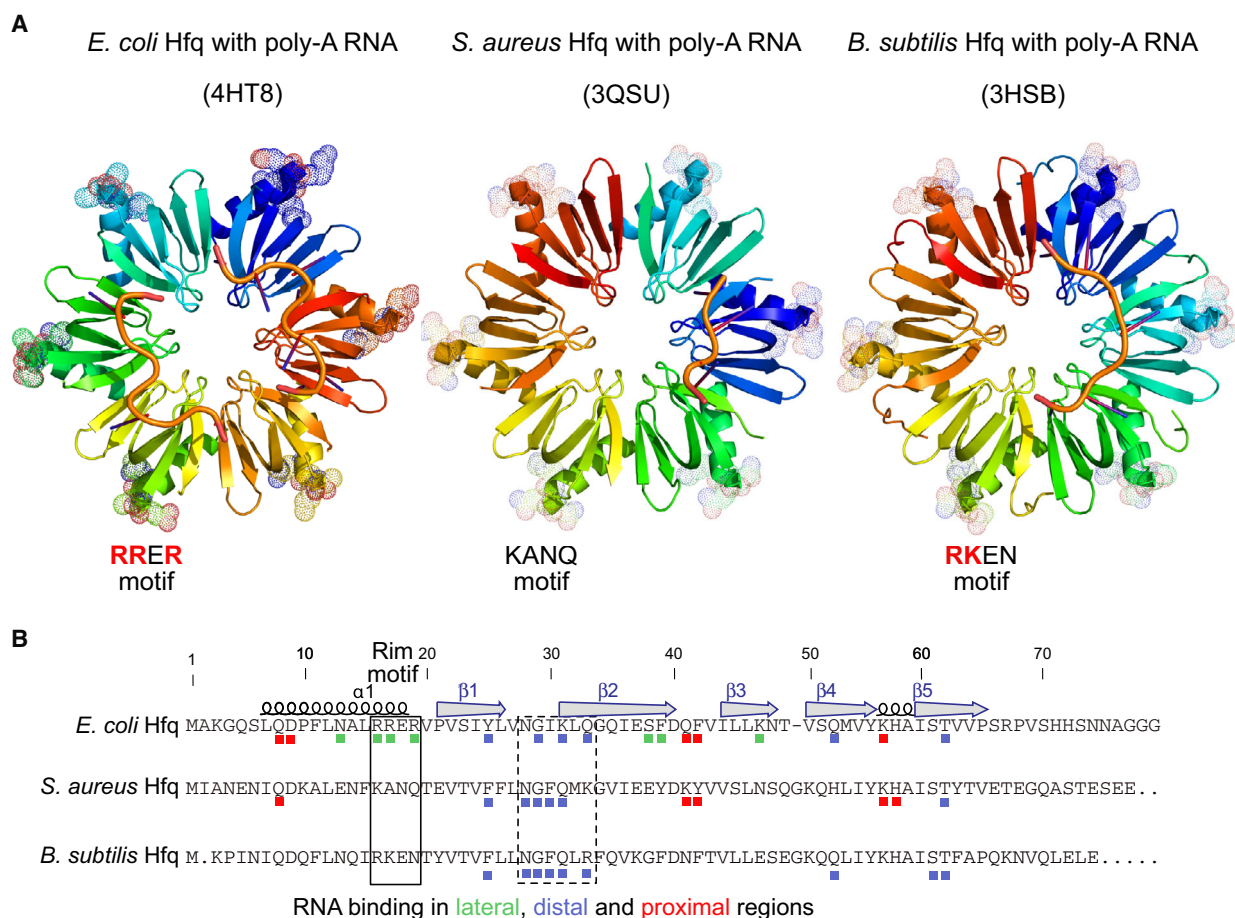


Fig. 1. Hfq binding to RNA differs between Gram-negative and Gram-positive bacteria. (A) Crystal structures of Hfq hexamers from the Gram-negative *E. coli* and Gram-positive *B. subtilis* and *S. aureus* bound to poly-A RNA [60,155,156]. The different monomers of Hfq are indicated in different colours. The location of the rim RNA-binding motifs in the hexamer is indicated with spheres. The arginine and lysine amino acids highlighted in red are essential for RNA binding. The images were generated using PyMol. (B) Sequence alignment of Hfq proteins from *E. coli*, *S. aureus* and *B. subtilis*. The structural sequence alignment was adapted from Stanek et al 2017 [157] and was generated using MAFFT [158] and MUSCLE [159]. Sequence accession numbers: *Escherichia coli* (BAE78173.1), *Staphylococcus aureus* (ADC37472.1), *Bacillus subtilis* (BAM57957.1). The location of the rim RNA-binding motif is indicated with a solid box. The dashed box indicates regions where there are differences in the way Hfq homologs interact with RNA (based on crystal structural studies). Structures showing RNA interactions with Hfq lateral regions were only available for *E. coli* and therefore are only shown for this organism. Similarly, interactions between *B. subtilis* proximal regions and RNA are also not available and therefore not highlighted. Residues previously described to contact the RNA on the rim (lateral), distal and proximal regions of Hfq are marked with green, blue and red boxes, respectively [156,157,160,161].

efficient when siRNAs (small interfering RNAs) with a relatively low GC content are used [51], supporting the idea that high GC content could be less favourable for sRNA-mediated regulation or it could be more dependent on RNA chaperones.

However, some features of Hfq cannot be explained by the above hypothesis. *E. coli* Hfq strand displacement and annealing activity were indeed shown to be strongly associated with the GC content of the RNA substrates *in vitro*. However, this had little to do with the thermodynamic properties of the duplex, but more

with the preference of Hfq for binding AU-rich sequence elements [52]. Also, in some GC-rich Gram-positive actinobacterial lineages, such as *Mycobacterium*, Hfq is completely absent [53]. In *Clostridium difficile*, a Gram-positive pathogen which has a low GC content of 29% (Table 1), Hfq plays diverse roles and has been proposed to regulate many genes involved in sporulation [54]. In *C. difficile*, Hfq is essential for normal growth and cell morphology, while its deletion reduces the stress tolerance and increases the ability of sporulation and biofilm

Table 1. Phenotypes of Hfq deletion strains in Gram-positive bacteria

Species	Strain	Key features	Hfq expression	Stress tolerance in Δhfq	Δhfq growth defects	Gene expression changes in Δhfq	Virulence of Δhfq	%GC
<i>Streptococcus pyogenes</i>	Hfq is absent [48]							38.4%
<i>Streptococcus pneumoniae</i>								39.6%
<i>Enterococcus faecium</i>								37.8%
<i>Bacillus subtilis</i>	BSB1 [59] 168 [6373]	Protein and mRNA detected under various conditions [59] Protein and mRNA slightly upregulated during transition to stationary phase [59,63,73] *Protein levels stable during transition to stationary phase in [63], in contrast to [73]	Reduced tolerance of stationary phase stress [59]	Survival defect only in stationary phase in rich medium [59]	Altered expression of a set of RNAs during stationary phase [59] including toxin/antitoxin RNAs [63,73]			43.6%
<i>Listeria monocytogenes</i>	EGD serotype 1/2a [39]	mRNA levels increased under specific stress conditions (e.g. osmotic and ethanol stress) [39]	Reduced tolerance to osmotic, ethanol stress and starvation [39] Acid, oxidative stress, thermotolerance and antibiotic resistance not affected [39]	No defects [39]	No data		Decreased pathogenicity in mouse infection studies [39]	37.9%
<i>Clostridium difficile</i>	630E [54]	High mRNA expression [54]	Reduced tolerance to oxygen and other stress factors [54]	Slower growth and morphological defects [54] Increased sporulation and biofilm formation [54]	Altered expression of genes involved in cell wall metabolism, sporulation, transcription regulation, stress response [54]			28.5%
<i>Staphylococcus aureus</i>	RN6390 [64,68,69] derived from NCTC 8325	Very low levels of mRNA detected [68]	Not affected [68]	No defects [68]	RNAIII and spa levels not affected [68] Stable pigment production [69]		Secreted virulence factors	32.7%

Table 1. (Continued).

Species	Strain	Key features	Hfq expression	Stress tolerance in Δhfq	Δhfq growth defects	Gene expression changes in Δhfq	Virulence of Δhfq	%GC
	COL [64,68,69] Newman [68,69]	Highly methicillin-resistant clinical isolate Virulent strain containing a high level of Clumping Factor	Protein not detected [64] Very low levels of mRNA detected [68].				expression not affected [68] Cytotoxicity not affected in RN6390 and COL [64]	
	NCTC8325-4 [64,69]	Derived from the clinical isolate NCTC8325, after curing for three prophages ($\Delta\phi 11$ $\Delta\phi 12$ $\Delta\phi 13$)	Very low levels of mRNA detected [64] Protein detected [64]	Increased tolerance to oxidative stress [64]	No defects [64]	Altered expression of genes, many related to stress and virulence [64] *Contradicting results on pigmentation: Increased in [64] / Stable in [69]	Reduced cytotoxicity and pathogenicity in mice [64]	

formation [54]. The altered expression of genes involved in cell wall metabolism, sporulation, stress response and transcription regulation in the *hfq* mutant shows that Hfq could play a crucial role for *C. difficile* physiology by participating in RNA regulation [54]. Hfq also appears to mediate sRNA regulation in *Listeria monocytogenes* that has a GC content of 38% (Table 1) [55,56]. Although *L. monocytogenes* Hfq is not essential for cell viability, it contributes to pathogenicity and its absence impairs adaptation to various stresses [39]. It interacts with several RNAs and is required for the interaction of the regulatory sRNA LhrA with its target RNAs (Fig. 2A) [55,56]. Thus, the correlation between GC content and the involvement of Hfq in sRNA-mediated regulation should be further investigated, as it appears that Hfq has various important roles in species with low and high GC content.

The functionality of Hfq homologues is often assessed by their ability to complement Hfq deletion phenotypes in *E. coli* or *Salmonella* [57,58]. Hfq from both *L. monocytogenes* and *C. difficile* is able to effectively replace *E. coli* Hfq, which supports a role in sRNA-mediated regulation [56,57]. However, *S. aureus* and *B. subtilis* Hfq cannot [58,59], implying that the functional differences between different species Hfqs are more likely to be linked to structural differences and how they interact with RNA (Fig. 1A). Interestingly, the RRER motif in *E. coli* Hfq that plays an important role in annealing activity is absent in *S. aureus* Hfq and not well conserved in *B. subtilis*. Both these Gram-positive Hfqs have poor annealing activity *in vitro* [47] (Fig. 1A). Therefore, this suggests that in *S. aureus* and *B. subtilis* Hfq may not be involved in mediating sRNA–RNA interactions. Furthermore, it has been suggested that sRNA–mRNA interactions are facilitated by Hfq binding to mRNAs and sRNAs, from the distal and proximal face, respectively (see Fig. 1B for interacting regions). However, RNA binding to the distal face of Hfq is different between Gram-negative and Gram-positive species: while in Gram-negative bacteria, the distal face contains tripartite binding motifs for poly-(ARN)_n sequences (R is purine, and N is any nucleotide), in Gram-positives there are bipartite binding motifs, interacting with poly-(AN)_n repeats [60]. Finally, Gram-positive Hfq proteins also lack the extended and structurally disordered C terminus found in *E. coli*, which also makes an important contribution to the Hfq chaperone function [61,62].

How could these structural differences affect the impact of Hfq on the transcriptome? Studies in *S. aureus* and *B. subtilis* have shown that deletion of Hfq does

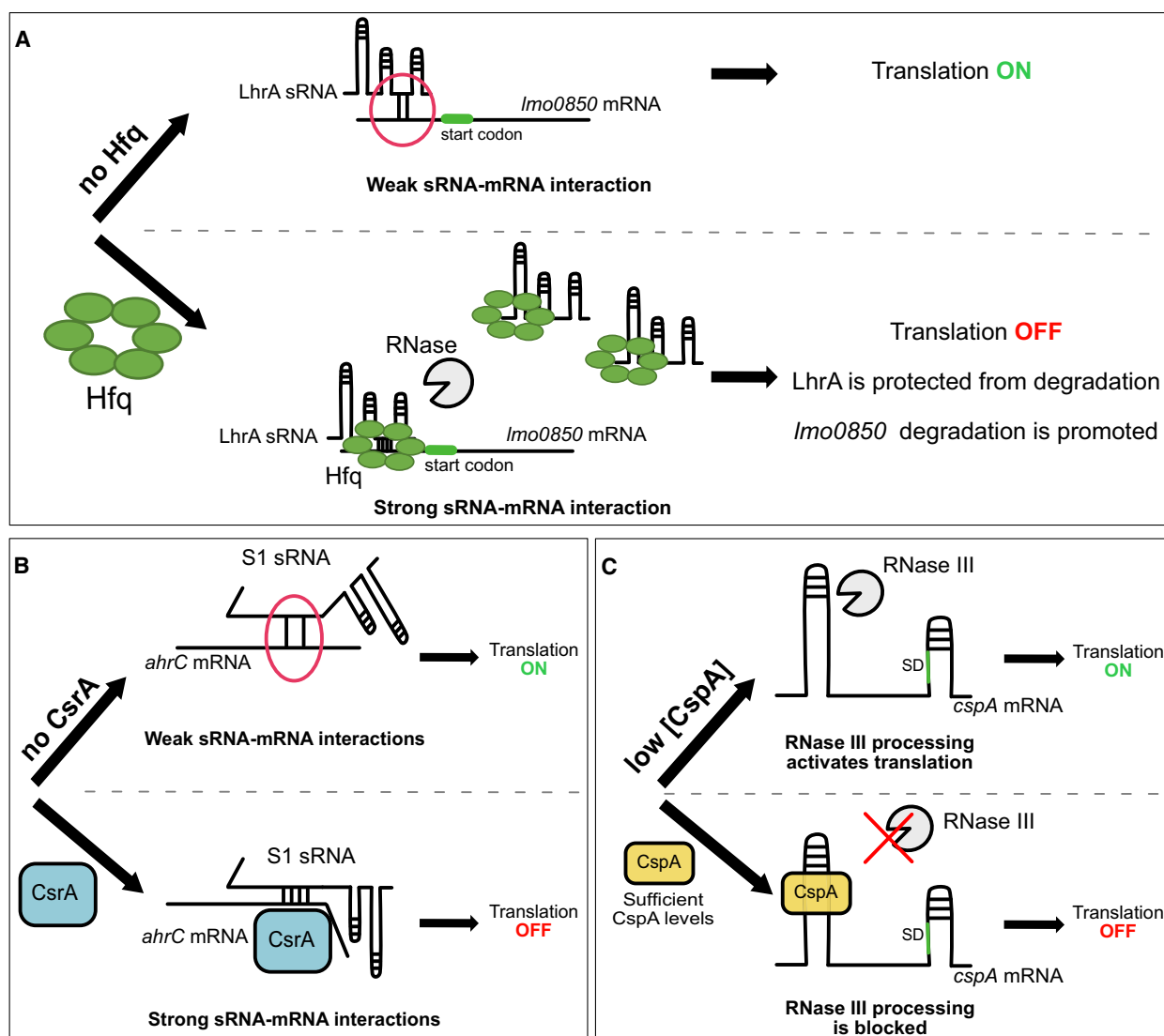


Fig. 2. Mechanisms of post-transcriptional regulation in Gram-positive bacteria. (A) In *L. monocytogenes*, Hfq stimulates the interaction between the LhrA sRNA and its target mRNA *lmo0850*, encoding a small hypothetical protein of unknown function. When Hfq is absent, the sRNA-mRNA interaction is not strong. In the presence of Hfq, LhrA is stabilized and its interaction with *lmo0850* is favoured, leading to inhibition of translation initiation and promoting the degradation of the target mRNA by an unidentified ribonuclease [56]. We can speculate that Hfq is a key player for sRNA-mediated regulation in *L. monocytogenes*, and since it is implicated in stress response and virulence [39], it may regulate the expression of virulence factors. (B) In *B. subtilis*, CsrA promotes the interaction between the SR1 sRNA and its target mRNA *ahrC* that encodes the transcriptional activator of the arginine catabolic operons *rocABC* and *rocDEF*. The base pairing of the RNAs inhibits the translation of the mRNA. If the SR1 levels are high, the complex is formed even in the absence of CsrA. CsrA binding to the *ahrC* mRNA is required for the efficient interaction and translation repression, while binding to the SR1 seems to have a minor role [76]. Model adapted from [76]. (C) In *S. aureus*, CspA regulated its own expression by a negative feedback mechanism. For the translation of *cspA* mRNA, processing by RNase III is necessary [101]. However, CspA binds the 5' UTR of its own mRNA, inhibiting RNase III processing and therefore downregulating its own expression [92]. Since CspA was found to bind and regulate – either positively or negatively – transcripts related to virulence and stress response, such as the alternative σ^B factor (positive regulation) and the cold-shock protein CspC [92], the model presented in the figure could be a generalized negative regulation mechanism, where CspA antagonizes with RNase III.

not impact the stability of many transcripts [59,63,64], and therefore, it is reasonable to assume that the loss of Hfq annealing activity in these species has resulted in a

less impactful role in co- and post-transcriptional regulation. Using global RNA-binding approaches such as UV cross-linking and immunoprecipitation experiments

(CLIP or CRAC [65,66]), it should be possible to figure out exactly what RNA Hfq directly binds in Gram-positives, which should help clarify to what extent the protein impacts the transcriptome and what pathways it directly regulates. Whether or not Hfq chaperones sRNA–RNA interactions in Gram-positives *in vivo* could be tested using global RNA proximity ligation approaches such as RIL-seq or CLASH [40,67].

The importance of *S. aureus* Hfq is also somewhat obscure, with several research articles presenting conflicting findings [64,68,69] (Table 1). Deletion of Hfq had no effect on growth of most laboratory and clinical *S. aureus* strains tested (summarized in Table 1) [64,68]. Although *S. aureus* Hfq has very poor annealing activity *in vitro* [47], the protein does interact with sRNAs and mRNAs, including the sRNA RNAIII, which is involved in regulating many virulence genes, and the *spa* mRNA, which encodes a protein that promotes immune suppression. However, in the strains studied (RN6390, Newman, COL), Hfq deletion does not affect the expression or stability of RNAIII and the *spa* mRNA [68,70]; therefore, it seems unlikely that the protein chaperones this interaction in *S. aureus* or this activity is not essential. Our impression is that Hfq could be more important in some clinically relevant strain backgrounds (see Table 1), but this needs to be investigated more thoroughly. Deleting Hfq in the *S. aureus* 8325-4 strain resulted in increased pigmentation and higher resistance to oxidative stress. It was proposed that the increased oxidative resistance was related to the higher levels of carotenoid pigments produced by the mutants, as these pigments act as antioxidants [64]. It is important to note that 8325-4 is a strain defective for the stress-associated sigma B factor (σ^B), due to a deletion in a positive regulator of σ^B , *rsbU* gene [71]. As a result, the carotenoid production in this strain is generally very low and it forms white colonies. However, a later study showed that Hfq deletion does not impact pigmentation in several strains, including 8325-4 [69] (Table 1). It is possible that some of the differences in the results could be explained by secondary mutations that somehow restored carotenoid pigment production in the 8325-4 Hfq deletion strain used by Liu and colleagues [64].

Impressively, in *S. aureus* and *B. subtilis* the impact of Hfq deletion was measured under nearly 2000 growth conditions [59,68] and *B. subtilis* Hfq only seemed to be important for survival in stationary phase in rich medium [59]. *B. subtilis* Hfq is not essential for sRNA-dependent post-transcriptional regulation, but it binds and stabilizes a small number of sRNAs [59,63,72,73]. Interestingly, another *Bacillus* species, *B. anthracis*, expresses three Hfq homologs. Two are

chromosome encoded (Hfq1 and Hfq2), and one is expressed from a virulence plasmid (Hfq3) [74]. Hfq2 has the closest protein sequence to the *B. subtilis* Hfq and forms the typical hexamer, while Hfq1 is a monomer. Although Hfq3 has the most divergent sequence compared with other *Bacillus* Hfqs, it can form hexamers and can partially complement Hfq function in *E. coli* [75]. Remarkably, its overexpression is toxic and leads to severe growth defects, a phenotype associated with residues on the distal face of the protein, which usually binds mRNA in other species [75].

In conclusion, the function as well as the importance of Hfq in *S. aureus* and many other Gram-positive bacteria remain a bit of mystery and clearly more detailed analyses need to be performed to clarify its role and significance in these microorganisms. Regardless, although Hfq is found in many Gram-positives, it does not seem to function in the same way or have a major impact on gene expression.

The role of CsrA in Gram-positive bacteria

As ProQ is absent in Gram-positive bacteria, and Hfq does not seem to play an equally important role as in Gram-negative bacteria [76], a major question in the field is whether Gram-positives employ or actually need general RNA chaperones for post-transcriptional regulation. For example, Gram-positives may simply utilize a diverse number of chaperones that have specificity towards some sRNA-target interactions. Alternatively, it is certainly possible that many sRNA-target interactions in Gram-positives may not need a chaperone as they could involve extensive base-pairing interactions that do not need to be stabilized or mediated by RNA chaperones. To better understand how sRNA–RNA interactions are regulated in Gram-positive bacteria, significant effort is being made to identify RBPs that could mediate RNA–RNA interactions (e.g. see [77]). One protein that has been proposed to play an important contribution in sRNA–RNA interactions in Gram-positive bacteria is CsrA.

The global post-transcriptional regulatory system carbon storage regulator/repressor of secondary metabolites (Csr/Rsm) is broadly conserved in bacteria and has been extensively studied in Gram-negative bacteria (reviewed in [78]). Its basic component, CsrA (or RsmA), is a global post-transcriptional regulator, involved in various aspects of the bacterial physiology, including motility, biofilm formation and virulence [78]. Structural studies of *E. coli* CsrA, which is a small homodimeric protein, have shown that it contains five β -strands and one α -helix, with the amino acids within the β 1 and β 5 strands contributing to

RNA binding [79–81]. CsrA has a strong preference for binding purine (AG)-rich RNA sequences in the 5'-UTR of target mRNAs, including Shine–Dalgarno (SD) sequences [66,82]. Binding of CsrA to the SD sequence can trigger degradation of the RNA by blocking ribosome access. However, CsrA can also enhance mRNA stability and promote translation, by protecting transcripts from RNase activity or preventing the formation of structures that would otherwise inhibit ribosome binding [1,83,84]. sRNAs, such as CsrB in *E. coli*, can bind (or sponge or sequester) CsrA and act as antagonists by preventing its association with target mRNAs [78].

The Csr/Rsm regulatory system plays an important role in pathogenicity, by regulating the expression of virulence factors in proteobacterial pathogens, such as *Pseudomonas*, *Salmonella* and pathogenic *E. coli* (reviewed in [78,84]). Putative homologs of CsrA have also been found in many Gram-positive bacteria [79]; however, the protein has only been studied in detail in *Clostridium* and *B. subtilis*. In *Clostridium acetobutylicum*, a species used commercially for the production of chemicals and biofuels, CsrA is involved in regulation of metabolic pathways such as flagella assembly, membrane transportation system, sporulation and central carbon metabolism [85]. A recent study in *C. difficile* showed that CsrA may contribute to carbon metabolism and also has a crucial role in virulence-associated processes, such as toxin production and motility [86].

In *B. subtilis*, CsrA is involved in flagella biosynthesis [83]. Until recently, the only target mRNA that had been identified was the *hag* mRNA that codes for the flagellin protein of *B. subtilis*. Similar to how CsrA controls gene expression in Gram-negative bacteria, *B. subtilis* CsrA binds to the *hag* mRNA Shine–Dalgarno sequence and causes translational repression. Although CsrA homologues are abundantly present in Gram-positive bacteria and appear to function in a similar way as in Gram-negatives, how the activity of CsrA is regulated in Gram-positives is different. As mentioned above, in the Gram-negative *E. coli* sRNAs regulate CsrA activity by sponging it or sequestering it [78]. In *B. subtilis*, however, the FliW protein plays the role of the CsrA antagonist. The interaction between these proteins is important for regulating flagellin biosynthesis [87]. FliW inhibits CsrA binding to target mRNAs using an allosteric noncompetitive mechanism [88]. FliS is a second chaperone also involved in the CsrA–FliW system of regulating flagellin production [89].

Recently, another role for CsrA in *B. subtilis* was discovered. CsrA binds both the small regulatory RNA SR1 and its target *ahrC* mRNA and enhances

their interaction. The *ahrC* mRNA encodes for a transcriptional activator of arginine catabolic operons. CsrA-mediated binding of SR1 to *ahrC* blocks ribosome binding and translation of the mRNA [76] (Fig. 2B). CsrA is necessary for the efficient pairing of the RNAs, and it is the only protein that has been found to enhance sRNA–mRNA interactions in Gram-positive species [76]. It was proposed that CsrA could be acting as a general chaperone that mediates coupling of sRNAs with their mRNA targets in Gram-positive bacteria; however, experimental evidence for this is still lacking.

Thus, it appears that CsrA affects some properties that are necessary for bacterial adaptation to harsh environments, such as motility and virulence factor production, in both Gram-negative and Gram-positive species. However, regulation of this protein activity seems to be different in these groups: sRNA antagonists of CsrA, which usually occur in γ -proteobacteria, have not been yet found in Gram-positives. Furthermore, phylogenetic analyses have shown that noncompetitive allosteric regulation of CsrA by a protein like *B. subtilis* FliW may also be present in other bacteria from both groups, except the γ -proteobacteria that lack the FliW protein [87]. In conclusion, all the available data imply that CsrA plays a more important role in post-transcriptional regulation than Hfq, in the Gram-positive species in which it has been studied, and that it may also contribute significantly to pathogenicity. Whether CsrA indeed consists a global regulator of gene expression in Gram-positive bacteria and whether it mediates more sRNA–RNA interactions remain to be elucidated.

Cold-shock proteins contribute to various stress responses

Another well-studied category of RBPs regulating bacterial adaptive responses is cold-shock proteins (CSPs). CSPs are a family of small proteins that are highly conserved in both sequence and structure. These DNA- and RNA-binding chaperones contain cold-shock domains (CSD) [90,91] and are widespread among all kingdoms of life [92]. Many CSPs, as their name suggests, are highly expressed in response to a decrease in temperature and help counteract the harmful effects of cold shock. For example, by acting as RNA chaperones and binding on mRNA, they prevent the formation of secondary structures in low temperatures, facilitating initiation of translation and therefore promoting the adaptation and survival to low temperatures [93]. The *E. coli* CspA, the first CSP that was discovered [94], preferentially binds single-stranded

pyrimidine-rich sequences on mRNAs, but it has also been reported to bind RNA hairpins [95]. The RNA binding is promoted by aromatic amino acid residues located on a positively charged surface formed by β -strands [95,96].

The CSPs have been most extensively studied in *E. coli*, which contains 9 CSPs, four of which are cold-inducible [90]. Homologous proteins have been classified as CSPs in many other bacterial species, including Gram-positive Firmicutes [97]. Three CSPs have been discovered in *B. subtilis*, *S. aureus*, *L. monocytogenes* and *Clostridium botulinum*. In *B. subtilis*, the three CSPs discovered are all cold inducible and the presence of at least one of them has been shown to be essential for viability [98]. However, none of the *L. monocytogenes* CSPs are essential for viability under optimal growth conditions, but CspA is critical for growth in the cold [99]. CSPs homologous genes have been classified into 5 different clades and 12 subclades, based on their phylogenetic distance [97]. CSPs of Gram-positive bacteria were classified in clade Ib, which are labelled as being involved in regulation of virulence, cold and osmotic shock resistance.

Cold-shock response is not the only function of CSPs: they also contribute to other adaptive responses, such as adaptation to oxidative and osmotic stress, host cell invasion and nutrient starvation [92,93,97,100]. Even though most of the studies on CSPs have focused on *E. coli* and *B. subtilis*, there are data supporting an important role for them in adaptation and stress tolerance in some other important Gram-positive species, as described below. This makes them appealing targets for the development of antimicrobials to inhibit bacterial adaptation and growth. A recent study in *S. aureus* [92] revealed that CspA is a global post-transcriptional regulator. It binds hundreds of transcripts *in vivo* and may therefore have a larger impact than Hfq on gene expression in this organism. Interestingly, the majority of the target mRNAs were reported to be processed by RNase III [92,101], implying a putative antagonistic role for CspA, in which it would inhibit the ribonuclease function, by binding the targeted mRNA structures (Fig. 2C) [92]. Among the transcripts bound by *S. aureus* CspA, many encode proteins involved in amino acid catabolism, nucleoside and carbohydrate synthesis, pathogenesis and adaptation to stress. This RNA chaperone can affect the stability and translation of its targets both positively and negatively and its deletion results in bacterial aggregation and lower resistance to oxidative stress [92]. Non-coding RNAs, such as sRNAs, were also identified among the chaperone's targets [92], but it remains unclear if CspA – like *E. coli* Hfq and CsrA – can also

mediate the coupling of sRNAs with their RNA substrates.

Like Hfq, *E. coli* CSPs can also bind DNA through the cold-shock domain (CSD; [102–104]) and this activity is conserved in Gram-positive bacteria. In *S. aureus*, CspA (MsaB) binds a promoter region of the *cap* operon *in vitro* to activate genes involved capsular polysaccharide formation, which is important for survival within the host [105]. CspA, together with the σ^B transcription factor and the staphyloxanthin operon, is also involved in a complicated regulatory network that involves both the RNA-binding activity and the DNA-binding activity of CspA. Staphyloxanthin, the carotenoid pigment that gives the yellow colour in *S. aureus*, is a major virulence factor that protects the bacteria against oxidative stress during the host infection, through its powerful antioxidant properties [106,107]. Deletion of CspA leads to reduced staphyloxanthin production and lower levels of σ^B factor, which is required for the expression of the staphyloxanthin biosynthetic operon (*crtOPQMN*) [92,107]. Thus, the reduced pigmentation in the *cspA* mutants is consistent with the reduced resistance to oxidative stress in these strains [92]. CspA was shown to bind the σ^B RNA transcript and upregulate its expression [92]. However, subsequently, CspA was reported to act as a transcriptional activator on the *crtOPQMN* operon as well, by directly binding to the promoter [108]. Interestingly, σ^B was not also required for this activation but also found to downregulate the transcription of *msaB* [108]. Therefore, by binding DNA and RNA, CspA has a major influence on controlling expression of genes involved in a variety of adaptive responses as well as controlling pathogenicity.

In *L. monocytogenes*, CSPs play a crucial role in efficient adaptation to cold, osmotic and oxidative stress [99,109]. By affecting the expression of genes involved in flagella biosynthesis and virulence, they promote host cell pathogenicity, cell aggregation and motility, properties contributing to survival in harsh conditions [99,109,110]. Flagella formation and motility were also observed to be affected by CSPs in the food pathogen *Clostridium botulinum* ATCC 3502 [111]. Its three CSPs are induced upon a temperature drop and contribute to cold-shock response, but several are also involved in adaptation to salt, pH and ethanol stress [111]. In the opportunistic pathogen *Enterococcus faecalis*, CSP CspR is upregulated during cold-shock and stationary phase and is required for virulence and efficient survival under stress conditions, such as nutrient deprivation [100,112].

In conclusion, like CsrA, CSPs are important players in post-transcriptional regulation of many

important Gram-positive pathogens, contributing significantly to their adaptation in different stressful environments and promoting their pathogenicity.

The role of atypical RNA-binding proteins regulating adaptive responses in Gram-positive bacteria

Having discussed the roles of well-characterized and canonical RBPs in adaptive responses in Gram-positive bacteria in some detail, we felt it was important to highlight some other interesting RBPs that in our opinion deserve to be more in the spotlight. This section discusses the roles of three RBPs that, at first glance, do not look like typical RBPs, as they are missing well-defined RNA-binding domains, but have received more attention recently as they play an important role in adaptation to harmful environments.

The DNA- and RNA-binding protein SpoVG, a global regulator of nutrient adaptation responses in Gram-positive bacteria

When nutrients become limiting, bacteria tend to exhibit many different behaviours to ensure their survival. This includes the production of antimicrobial compounds, formation of biofilms and in some species, such as *C. difficile* and *B. subtilis*, sporulation. Since sporulation renders bacteria almost impenetrable to antibiotics, this pathway is being extensively studied, as blocking this process may enable us to find a way to combat bacterial antibiotic resistance. Research on sporulation in *B. subtilis* led to the identification of SpoVG, a protein that is highly conserved and in Eubacteria [113–115]. In *B. subtilis*, SpoVG plays an important – but not essential – role in sporulation, by regulating asymmetric septation and promoting cortex formation [114,116]. Lack of SpoVG does not cause severe defects, unless SpoIIB, another protein participating in the engulfment stage of sporulation [117], is also absent, something that shows synergistic action and redundancy of these proteins in spore formation [117,118]. SpoVG is also involved in haemolytic activity caused by *B. subtilis* [119]. Recently, it was also shown to be essential for the formation of *B. anthracis* spores, which consist the infectious form of this bacterium [118]. The absence of SpoVG completely impaired its sporulation capabilities, with the inhibition occurring before the asymmetric division step, indicating that the *B. anthracis* SpoVG has a different role than it does in *B. subtilis* [118].

Is SpoVG therefore only relevant to spore-forming bacteria? The answer is no: in *S. aureus*, SpoVG is

involved in antibiotic resistance [120,121], virulence [122,123] and cell aggregation [124]. In the strain N315, it contributes to cell wall biosynthesis and antibiotic resistance, by binding to the promoter and controlling the expression of genes participating in oxacillin resistance and cell wall metabolism [120]. SpoVG also controls the expression of virulence factors Spa and clumping factor B (ClfB) both by binding to the promoters of their genes and by regulating Rot, a regulatory protein that controls the above virulence factors [123]. Moreover, SpoVG was shown to positively regulate the ability of *S. aureus* to bind human fibrinogen [123] and negatively regulate cell aggregation, by downregulating the expression of SasC, a surface adhesin [124]. In conclusion, all the available data suggest that SpoVG could be a key contributor in regulating a wide variety of adaptive responses.

One of the many reasons why SpoVG is such an interesting protein is that, like CspA and Hfq, it binds both DNA and RNA [115,123,125,126], implying a role as a transcription factor and a post-transcriptional regulator. This dualistic function makes it possible to connect multiple regulatory networks and may allow much finer control of gene expression.

In *Borrelia burgdorferi*, a Gram-negative bacterium with an atypical Gram-negative cell membrane [127,128], SpoVG was found to bind RNA *in vitro*. Here, SpoVG binds both its own transcript and its own gene, suggesting a negative feedback mechanism, where the protein controls its expression both at the transcriptional and post-transcriptional levels [126]. To the best of our knowledge, this type of regulation where a protein regulates its own transcription and mRNA translation is rare in bacteria. SpoVG associates with transcripts that encode proteins involved in glycerol metabolism and host colonization, suggesting that it may affect their expression and influence the adaptation to different environments [126]. However, these interactions need to be further studied, in order to understand in which way SpoVG affects the expression of its target RNAs.

In vitro RNA-binding activity was also observed for *L. monocytogenes* SpoVG. The absence of SpoVG increased lysozyme resistance and virulence, while causing defects in bacterial motility [125]. Rli31, a sRNA involved in lysozyme resistance, was found to bind both SpoVG protein and its mRNA 5'-UTR *in vitro*, however without affecting *spoVG* mRNA or protein abundance [125]. SpoVG was also shown to bind multiple RNA molecules *in vitro*, and its RNA-binding affinity was higher than its DNA-binding affinity [125]. Unpublished crystallographic studies on *B. subtilis* SpoVG revealed that *in vitro*, like Hfq, it can form hexamers [129].

Although there is still much to be learned about the DNA- and RNA-binding properties of SpoVG *in vivo*, the above findings do suggest that SpoVG could be a major player in regulating adaptive responses on multiple levels, which warrants further investigations. Since SpoVG is widely conserved between bacterial species [115], it is logical to assume that it also binds RNA in other Gram-positive bacteria. Whether or not SpoVG uses the same domains for binding DNA and RNA remains unclear. How SpoVG is recruited to RNA and whether it has any sequence specificity are also not known.

Proteomic studies suggest that almost half of the identified human RBPs could have dual DNA- and RNA-binding capability (DRBPs) and are linked to a multitude of processes, including many stress-responsive proteins involved in DNA repair and transcription [8,130]. However, while many examples have been described in higher eukaryotes, DRBPs are still understudied in bacteria. Other notable examples of bacterial DRBPs include the *S. aureus* transcription factor SarA [131], the *E. coli* transcriptional repressor H-NS [132], which regulates the decay of a selected number of RNAs and *S. aureus* CspA (as mentioned above). The biological significance of SarA RNA-binding activity is not completely clear yet; however, cells lacking SarA showed altered mRNA decay properties of over 100 transcripts [131,133], implying a role for SarA in regulating RNA turnover. Excitingly, recent proteomic studies also imply that many more DRBPs may exist in bacteria [11,12,134], and therefore, we expect that many more examples of bacterial DRBPs will be described in the near future.

Bacterial RBPs can manipulate the host

Quite possibly the most stressful environment for bacteria is the host environment. Here, invading microorganisms need to find ways to extract essential nutrients from an otherwise nutrient-poor environment. In addition, they are constantly attacked by the host immune system. What is really fascinating is that bacteria have developed very sophisticated approaches to evade the host immune system and even thrive within host cells. The most obvious way to adapt to such an environment would be to employ a combination of transcription factors, RBPs and other regulatory molecules to remodel your own transcriptome and proteome to make the cell's physiology more compatible. Alternatively, you could use RBPs to manipulate the host environment. A number of pathogenic bacteria secrete effector molecules that act as virulence factors and generally target host proteins to interfere with the host

cellular functions [135]. So how is this relevant to regulation of gene expression?

One possible way to manipulate the host's response to the invasion is to use bacterial RNAs and RBPs. Much of the material that is secreted by bacteria is contained within vesicles that are packed with proteins and RNA. Evidence that sRNAs play a role in host-pathogen communication in Gram-negative bacteria was recently provided [136–138]. sRNA-mediated regulation is usually controlled by RBPs; therefore, one might expect to find these in secreted vesicles as well. However, a recent large-scale study showed that secreted effectors in Gram-negative bacteria generally do not contain conserved RNA-binding domains, suggesting that if effector proteins target RNA to manipulate host gene expression post-transcriptionally, they probably use novel RNA-binding domains [139]. The first bacterial secreted RBP was recently identified in the Gram-positive pathogen *L. monocytogenes*, and, indeed, it does not contain a canonical RNA-binding domain [140].

Protein Lmo2686, which has been named after an ancient Greek goddess (Zea), is a small RBP that is associated with a subset of the pathogen's RNAs and triggers the host cell immune response. Zea forms a homohexamer, like Hfq, and binds a distinct set of *L. monocytogenes* RNAs in the extracellular environment. When *L. monocytogenes* infects mammalian cells, Zea is secreted into the host's cytoplasm where it interacts with RIG-I (retinoic acid-inducible gene-I), a cytoplasmic sensor of viral RNA [141] that can induce the type I interferon (IFN) response [140]. Zea modulates the RIG-I-dependent signalling, and this strongly depends on the Zea-bound *L. monocytogenes* RNA molecules. Thus, the current model is that this RBP helps bacterial RNAs to act as effector molecules to induce the host innate response by delivering them to the RIG-I receptor [140].

Since the pathogen must escape the host's immune system in order to survive, it may seem that activating the host response does not offer any benefit. The activation of macrophages by IFN- γ , during type II IFN response, renders them capable of battling intracellular pathogens, like *L. monocytogenes*. However, during type I IFN response, induced by *L. monocytogenes*, IFN- $\alpha\beta$ production inhibits the macrophage activation by IFN- γ , and in this way, it increases the host susceptibility [142]. Therefore, by interacting with RIG-I receptor and activating the type I IFN response, Zea may play a critical role for creating a more favourable environment for the survival of *L. monocytogenes* inside the host. It is tempting to

speculate that similar mechanisms may be employed by other Gram-positive pathogens to manipulate the host response for their own benefit. For example, induction of the host's type I IFN response by *S. aureus* has been shown to promote its virulence [143]. During lung infection, *S. aureus* induces the type I IFN response in dendritic cells, by activating the receptor TLR9 (Toll-like receptor 9). The signal molecule recognized by TLR9 is staphylococcal DNA [144]. Thus, it is logical to assume that other signalling pathways also may exist, involving bacterial RNA as the signalling molecule that triggers the host immune response and RBPs are likely to assist the function of these 'signalling' RNA.

Zea is absent from nonpathogenic *Listeria* species, which supports a role in virulence. However, Zea orthologs were identified in other nonpathogenic bacteria, mainly of the genus *Bacillus*, that are rarely associated with disease [140]. The exact role of Zea in host–pathogen communication remains to be elucidated. Nevertheless, this first report of a secreted bacterial RBP transferring RNA that modulates the host immune response can pave the road for the discovery of a potentially conserved bacterial pathway for extracellular RNA that contributes to adaptation in hostile environments.

Alarmone synthetases as RNA-binding proteins: connecting post-transcriptional regulation with stress metabolism

Small alarmone synthetases (SASs) belong to an important group of stress-related bacterial proteins and recently came into the spotlight as RBPs [145]. Many interesting questions about SAS function have been raised [146], and therefore, we would like to briefly refer to this group of proteins, as we believe these factors could provide a direct link between sensing stress and regulation of gene expression.

SASs are widely distributed bacterial enzymes that belong to the RelA/SpoT homolog (RSH) protein family and synthesize the alarmone nucleotides guanosine tetraphosphate and pentaphosphate ((p)ppGpp) [147,148]. These signalling nucleotides regulate bacterial growth, pathogenicity and adaptation to stress, such as osmotic and antibiotic stress. Under stressful conditions that affect the cell wall, like exposure to cell wall-targeting antibiotics, SASs are overexpressed and (p)ppGpp is overproduced, helping the bacteria to overcome the stress [149,150]. As mentioned in the introduction, a lot of enzymes have been found to exhibit RNA-binding activity. Recently, a SAS enzyme was also shown to bind RNA.

In *B. subtilis* and *E. faecalis*, (p)ppGpp binds to the tetrameric SAS RelQ and allosterically activates its catalytic function [151,152]. Recently, it was discovered that RelQ of *E. faecalis* also binds single-stranded RNA in a sequence-specific manner [145]. The binding of RNA inhibits the enzymatic function, with the inhibitory effect being stronger when the RNA contains GG elements of the Shine–Dalgarno sequence [145]. RNA binding is not compatible with binding of (p)ppGpp on RelQ, something that could be due to binding on the same site. Under stress conditions, when (p)ppGpp levels are high, the synthetase activity of RelQ is allosterically activated and more (p)ppGpp is produced, while the RNA targets are released. In the absence of (p)ppGpp, RelQ binds RNA and possibly regulates its function, while the enzymatic activity is inhibited. The tetrameric structure of the enzyme is essential for this regulatory mechanism [145].

Although the RNA binding on RelQ has a negative effect on the enzymatic activity, the specific interactions need to be identified *in vivo* and their biological significance remains to be further studied. As suggested by Hauryliuk and Atkinson [146], the RNA binding may control the transformation of the enzyme from an active form, in which (p)ppGpp can allosterically induce the enzymatic function, to an inactive state, in which the RNA blocks (p)ppGpp binding and inhibits the enzymatic activity [146]. We can also speculate that at the same time, the enzyme, acting as an RBP, may have a regulatory effect on the bound RNAs. For example, in a hypothetical model in which the bound RNAs have a role in stress response – either by producing or regulating the production of stress response factors – when (p)ppGpp levels are low, RelQ will bind and block the RNAs, inhibiting the overproduction of stress response factors. Under stressful conditions, though, (p)ppGpp will bind on RelQ, inducing its own synthesis and releasing the RNAs that will be free to contribute to the stress response. In this way, the RNA–enzyme interactions could have a double regulatory effect.

Since SAS enzymes are broadly distributed, this RNA-binding regulatory mechanism could be widely distributed among bacteria [146]. However, it is clear not all the SAS enzymes are regulated in the same manner and there is strong species-specific variation. Surprisingly, while *S. aureus* RelQ is also allosterically activated by (p)ppGpp, its catalytic function remains unaffected by the same RNA oligomers that inhibit RelQ activity in *E. faecalis* [153]. Moreover, *S. aureus* SAS RelP is strongly inhibited, and not activated, by (p)ppGpp, while its enzymatic activity is not affected by RNA [154]. It was observed that the allosteric

binding site of (p)ppGpp is absent in RelP, which explains why (p)ppGpp does not have the same stimulating effect on the enzymatic activity and suggests that the catalytic inhibition is caused by orthosteric binding [154]. To conclude, it appears that even though SASs from different Firmicute species have similar regulatory mechanisms, there are small structural and functional differences that also lead to different regulation of their activity.

Conclusions and future perspectives

It is clear that RBPs play an essential role in regulating bacterial gene expression and allowing rapid adaptation to changing environments. As described above, despite impressive progress over the past few years in studying post-transcriptional regulation in Gram-positive bacteria, there is still a lot of catching-up to do. The discrepancy of the roles that well-studied RBPs play in Gram-negative and Gram-positive bacteria reveals that Gram-positives may use different and perhaps even more diverse mechanisms for post-transcriptional gene regulation and these may involve atypical RBPs. Since many Gram-positive species are important pathogens, studying how these organisms manage to rapidly adjust their gene expression in response to environmental changes and what molecular mechanisms they use to adapt to the host environment and battle the immune system can help us develop strategies to block these adaptive responses and combat the infections.

As mentioned in the introduction, a number of very powerful high-throughput technologies have recently been developed that allow global identification of protein–RNA and RNA–RNA interactions in diverse organisms and environmental conditions. Therefore, we anticipate that in the next few years, many studies will be published describing new regulators and novel interactions. It would be interesting to learn whether these applications can also be applied under infection conditions as this would allow us to further dissect the communication between pathogens and their hosts.

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Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

NC and SG conceived the review topic. Both authors also made the figures and wrote the manuscript.

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